PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

B2

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 5/16, A61K 48/00, C12M 3/00	A1	(11) International Publication Number: WO 97/25414 (43) International Publication Date: 17 July 1997 (17.07.97)

<table>
<tr>
<td>

(21) International Application Number: PCT/US97/00303

(22) International Filing Date: 10 January 1997 (10.01.97)

(30) Priority Data:
08/587,376   12 January 1996 (12.01.96)   US
08/712,111   13 September 1996 (13.09.96)   US

(71)(72) Applicant and Inventor: VANDENBURGH, Herman, H.
[US/US]; 135 Prospect Street, Providence, RI 02906 (US).

(74) Agents: FRASER, Janis, K. et al.; Fish & Richardson P.C., 225
Franklin Street, Boston, MA 02110-2804 (US).

</td>
<td>

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR,
BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE,
HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS,
LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL,
PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA,
UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB,
GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ,
CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

</td>
</tr>
</table>

(54) Title: DELIVERY OF BIOACTIVE COMPOUNDS TO AN ORGANISM

(57) Abstract

Disclosed herein is a method of delivering a bioactive compound to an organism that involves growing individual cells *in vitro* under conditions that allow the formation of an organized tissue, at least a subset of the cells containing a foreign DNA sequence which mediates the production of the bioactive compound; and implanting to organized tissue into the organism, whereby the bioactive compound is produced and delivered to the organism. Also disclosed herein is an *in vitro* method for producing a issue having *in vivo*-like gross and cellular morphology that involves providing precursor cells of the tissue; mixing the cells with a solution of extracellular matrix components to create a suspension; placing the suspension in a vessel having a three-dimensional geometry approximating the *in vivo* gross and cellular morphology of the tissue and having attachment surfaces coupled thereto, allowing the suspension to coalesce; and culturing the cells under conditions in which the cells form an organized tissue connected to the attachment surfaces. Also disclosed herein is an apparatus for producing *in vitro* a tissue having *in vivo*-like gross and cellular morphology. This apparatus includes a vessel having a three-dimensional geometry approximating the *in vivo* morphology of the tissue and tissue attachment surfaces coupled thereto.

- 1 -

DELIVERY OF BIOACTIVE COMPOUNDS TO AN ORGANISM
Inventor: Herman H. Vandenburgh
Background of the Invention

This invention relates to the delivery of
5 bioactive compounds to an organism, and in particular to
methods and apparatus for the delivery of bioactive
compounds by implanting into the organism an organized
tissue producing the compounds.

One of the primary therapies used to treat disease
10 is the delivery of bioactive compounds to the affected
organism. Bioactive compounds may be delivered
systemically or locally by a wide of variety of methods.
For example, an exogenous source (i.e., produced outside
the organism treated) of the bioactive compound may be
15 provided intermittently by repeated doses. The route of
administration may include oral consumption, injection,
or tissue absorption via topical compositions,
suppositories, inhalants, or the like. Exogenous sources
of the bioactive compound may also be provided
20 continuously over a defined time period. For example,
delivery systems such as pumps, time-released
compositions, or the like may be implanted into the
organism on a semi-permanent basis for the administration
of bioactive compounds (e.g., insulin, estrogen,
25 progesterone, etc.).

The delivery of bioactive compounds from an
endogenous source (i.e., produced within the organism
treated) has also been attempted. Traditionally, this
was accomplished by transplanting, from another organism,
30 an organ or tissue whose normal physiological function
was the production of the bioactive compound (e.g., liver
transplantation, kidney transplantation, or the like).
More recently, endogenous production by cells of the
affected organism has been accomplished by inserting into
35 the cells a DNA sequence which mediates the production of

- 2 -

the bioactive compound. Commonly known as gene therapy,
this method includes inserting the DNA sequence into the
cells of the organism *in vivo*. The DNA sequence persists
either transiently or permanently as an extra-chromosomal
5 vector (e.g., when inserted by adenovirus infection or by
direct injection of a plasmid) or integrates into the
host cell genome (e.g., when inserted by retrovirus
infection). Alternatively, the DNA sequence may be
inserted into cells of the host tissue or an another
10 organism *in vitro*, and the cells subsequently
transplanted into the organism to be treated.

Summary of the Invention

In general, the invention features a method of
delivering a bioactive compound to an organism. The
15 method includes the steps of growing a plurality of cells
in vitro under conditions that allow the formation of an
organized tissue, at least a subset of the cells
containing a foreign DNA sequence which mediates the
production of the bioactive compound, and implanting the
20 cells into the organism, whereby the bioactive compound
is produced and delivered to the organism.

In a preferred embodiment of this method, the step
of growing may include mixing the cells with a solution
of extracellular matrix components to create a
25 suspension, placing the suspension in a vessel having a
three-dimensional geometry approximating the *in vivo*
gross morphology of the tissue and having tissue
attachments surfaces thereon, allowing the suspension to
coalesce, and culturing the coalesced suspension under
30 conditions in which the cells connect to the attachment
surfaces and form a tissue having an *in vivo*-like gross
and cellular morphology.

In other preferred embodiments, the DNA sequence
encodes the bioactive compound; the DNA sequence encodes

- 3 -

a protein which mediates the production of the bioactive
compound (for example, by regulating its expression or
encoding an intermediate to the bioactive compound); the
DNA sequence mediates the production of two bioactive
5 compounds; the tissue includes skeletal muscle; the
tissue includes myotubes; the bioactive compound is a
growth factor (for example, human growth hormone); the
bioactive compound is a bone morphogenetic protein; the
bone morphogenetic protein is BMP-6; the organized tissue
10 is implanted into the tissue of origin of at least one of
the cells; the cells include a first and a second
population of cells, at least a subset of each of the
populations containing a foreign DNA sequence which
mediates the production of a bioactive compound; the
15 foreign DNA sequence of the first population mediates the
production of a bioactive compound different from the
foreign DNA sequence of the second population; and the
foreign DNA sequence of the first population encodes a
bone morphogenetic protein and the foreign DNA sequence
20 of the second population includes a parathyroid hormone.

     In other preferred embodiments, the method
includes: the step of removing the organized tissue from
the organism to terminate delivery of the bioactive
compound; following the removal step, the step of
25 culturing the organized tissue *in vitro* under conditions
which preserve its *in vivo* viability; following the
culturing step, the step of reimplanting the organized
tissue into the organism to deliver the bioactive
compound to the organism; the step of isolating primary
30 cell types of at least one of the cell types of the
tissue; and the step of utilizing immortalized cells of
at least one of the cell types of the tissue.

     In other preferred embodiments of this method, the
tissue comprises substantially post-mitotic cells; during
35 the growing step, a force is exerted substantially

- 4 -

parallel to a dimension of the tissue; the force is
exerted on the individual cells during growth *in vitro*
and on the organized tissue during implantation *in vivo*;
the coalesced suspension exerts a force on the cells
5   substantially parallel to a dimension of the vessel; the
cells are aligned substantially parallel to a dimension
of the vessel; the vessel is substantially semi-
cylindrical in shape; the attachment surfaces are
positioned at opposite ends of the vessel; the alignment
10 is mediated by forces exerted by the coalesced
suspension; the cells comprise myotubes; the organism is
a mammal; and the mammal is a human.

In a related aspect, the invention features an
organized tissue producing a bioactive compound, the
15 tissue is produced by the steps of mixing a plurality of
cells with a solution of extracellular matrix components
to create a suspension, at least a subset of the cells
containing a foreign DNA sequence which mediates the
production of a bioactive compound; placing the
20 suspension in a vessel having a three dimensional
geometry approximating the *in vivo* gross morphology of
the tissue, the vessel having attachment surfaces
thereon; allowing the suspension to coalesce; and
culturing the coalesced suspension under conditions in
25 which the cells connect to the attachment surfaces and
form a tissue having an *in vivo*-like gross and cellular
morphology.

In a related aspect, the invention features an
organized tissue producing a bioactive compound. The
30 organized tissue includes a plurality of cells, grown *in
vitro* under conditions that allow the formation of an
organized tissue, and a foreign DNA sequence mediating
the production of a bioactive compound. The DNA sequence
is inserted into at least a subset of the cells. Also
35 included in the invention are organized tissues producing

a bioactive compound, the tissue being produced by any of the methods described herein.

In preferred embodiments, the organized tissue is skeletal muscle.

5   In a related aspect, the invention features an *in vitro* method for producing a tissue having an *in vivo*-like gross and cellular morphology. The method includes providing precursor cells of the tissue; mixing the cells with a solution of extracellular matrix components to
10   create a suspension; placing the suspension in a vessel having a three-dimensional geometry approximating the *in vivo* gross morphology of the tissue, the vessel having tissue attachment surfaces thereon; allowing the suspension to coalesce; and culturing the cells under
15   conditions in which the cells form an organized tissue connected to the attachment surfaces.

In preferred embodiments of this method, the step of providing includes isolating primary cells of at least one of the cell types which make up the tissue or
20   includes utilizing immortalized cells of at least one of the cell types which make up the tissue; the step of providing includes inserting a foreign DNA sequence into at least one of the cells which make up the tissue; the tissue includes substantially post-mitotic cells; the
25   coalesced suspension exerts a force on the cells substantially parallel to a dimension of the vessel; the cells are aligned substantially parallel to a dimension of the vessel; the vessel is substantially semi-cylindrical in shape; and the attachment surfaces are
30   positioned at opposite ends of the vessel.

In other preferred embodiments of this method, the DNA sequence encodes the bioactive compound; the DNA sequence encodes a protein which mediates the production of the bioactive compound; the DNA sequence mediates the
35   production of two bioactive compounds; the bioactive

- 6 -

compound is a growth factor; the organized tissue is
implanted into the organism, whereby the bioactive
compound is produced and delivered to the organism; and
the organized tissue is implanted into the tissue of
5 origin of at least one of the cells.

In a related aspect, the invention features an
organized tissue produced by the steps of providing
precursor cells of the tissue; mixing the cells with a
solution of extracellular matrix components to create a
10 suspension; placing the suspension in a vessel having a
three-dimensional geometry approximating the *in vivo*
gross morphology of the tissue, the vessel having tissue
attachment surfaces thereon; allowing the suspension to
coalesce; and culturing the cells under conditions in
15 which the cells form an organized tissue connected to the
attachment surfaces. Also included in the invention are
organized tissues produced by any of the methods
described herein.

In a related aspect, the invention features an
20 apparatus for producing a tissue *in vitro* having an *in
vivo*-like gross and cellular morphology. The apparatus
includes a vessel having a three-dimensional geometry
approximating the *in vivo* gross morphology of the tissue
and having tissue attachment surfaces in the vessel.

25   In preferred embodiments of this aspect of the
invention, the apparatus further includes a culture
chamber in which the vessel may be submerged; the vessel
is substantially semi-cylindrical in shape; the
attachment surfaces are coupled to opposite ends of the
30 semi-cylindrical vessel; the coalesced suspension exerts
a force on the cells substantially parallel to a
dimension of the vessel; and the cells are aligned
substantially parallel to a dimension of the vessel.

In a related aspect, the invention features a
35 method of regulating bone formation in an organism. The

- 7 -

method includes the steps of growing a plurality of cells
in vitro under conditions that allow the formation of an
organized tissue, at least a subset of the cells
containing a foreign DNA sequence which mediates the
5 production of a bone morphogenetic protein, and
implanting the tissue into the organism, whereby the bone
morphogenetic protein is produced and delivered to
chondroblastic or osteoblastic precursor cells.

In a preferred embodiment of this method, the step
10 of growing may include mixing the cells with a solution
of extracellular matrix components to create a
suspension; placing the suspension in a vessel having a
three-dimensional geometry approximating the *in vivo*
gross morphology of the tissue and having tissue
15 attachments surfaces thereon; allowing the suspension to
coalesce; and culturing the coalesced suspension under
conditions in which the cells connect to the attachment
surfaces and form a tissue having an *in vivo*-like gross
and cellular morphology.

20   In other preferred embodiments, the DNA sequence
encodes the bone morphogenetic protein; the DNA sequence
encodes BMP-6; the DNA sequence encodes a protein which
mediates the production of the bone morphogenetic protein
(for example, by regulating its expression or encoding an
25 intermediate to the bioactive compound); the DNA sequence
also mediates the production of another bioactive
compound; the tissue includes skeletal muscle; the tissue
includes myotubes; the bioactive compound is a growth
factor (for example, human growth hormone); the organized
30 tissue is implanted into the tissue of origin of at least
one of the cells; the cells include a first and a second
population of cells, at least a subset of each of the
populations containing a foreign DNA sequence which
mediates the production of a bioactive compound; the
35 foreign DNA sequence of the first population mediates the

- 8 -

production of a bioactive compound different from the
foreign DNA sequence of the second population; and the
foreign DNA sequence of the first population encodes a
bone morphogenetic protein and the foreign DNA sequence
5 of the second population includes a parathyroid hormone.

In other preferred embodiments, the method
includes: the step of removing the organized tissue from
the organism to terminate delivery of the bone
morphogenetic protein; following the removal step, the
10 step of culturing the organized tissue *in vitro* under
conditions which preserve its *in vivo* viability;
following the culturing step, the step of reimplanting
the organized tissue into the organism to deliver the
bone morphogenetic protein to the organism; the step of
15 isolating primary cell types of at least one of the cell
types of the tissue; and the step of utilizing
immortalized cells of at least one of the cell types of
the tissue.

In other preferred embodiments of this method, the
20 tissue comprises substantially post-mitotic cells; during
the growing step, a force is exerted substantially
parallel to a dimension of the tissue; the force is
exerted on the individual cells during growth *in vitro*
and on the organized tissue during implantation *in vivo*;
25 the coalesced suspension exerts a force on the cells
substantially parallel to a dimension of the vessel; the
cells are aligned substantially parallel to a dimension
of the vessel; the vessel is substantially semi-
cylindrical in shape; the attachment surfaces are
30 positioned at opposite ends of the vessel; the alignment
is mediated by forces exerted by the coalesced
suspension; the cells comprise myotubes; the organism is
a mammal; and the mammal is a human.

As used herein, by a "bioactive compound" is meant
35 a compound which influences the biological structure,

function, or activity of a cell or tissue of a living
organism.

By "bone morphogenetic protein" is meant an
extracellular osteogenic-stimulating molecule belonging
5   to the TGF-β superfamily. Bone morphogenetic proteins
("BMP") include a large number of proteins, for example,
BMP-2, -3, -4, -5, -6, -7, -11, and -12. Bone
morphogenetic proteins control the cellular events
associated with bone and cartilage formation and repair
10 (e.g., cellular growth, proliferation, and
differentiation). For example, bone morphogenetic
proteins alter the differentiation pathway of mesenchymal
cells towards the chondroblastic or osteoblastic lineage.

By "organized tissue" or "organoid" is meant a
15 tissue formed in vitro from a collection of cells having
a cellular organization and gross morphology similar to
that of the tissue of origin for at least a subset of the
cells in the collection. An organized tissue or organoid
may include a mixture of different cells, for example,
20 muscle, fibroblast, and nerve cells, but must exhibit the
in vivo cellular organization and gross morphology of a
tissue including at least one of those cells, for
example, the organization and morphology of muscle
tissue.

25   By "in vivo-like gross and cellular morphology" is
meant a three-dimensional shape and cellular organization
substantially similar to that of the tissue in vivo.

By "extracellular matrix components" is meant
compounds, whether natural or synthetic compounds, which
30 function as substrates for cell attachment and growth.
Examples of extracellular matrix components include,
without limitation, collagen, laminin, fibronectin,
vitronectin, elastin, glycosaminoglycans, proteoglycans,
and combinations of some or all of these components

- 10 -

(e.g., Matrigel", Collaborative Research, Catalog No.
40234).

By "tissue attachment surfaces" is meant surfaces
having a texture, charge or coating to which cells may
5   adhere in vitro. Examples of attachment surfaces
include, without limitation, stainless steel wire,
VELCRO", suturing material, native tendon, covalently
modified plastics (e.g., RGD complex), and silicon rubber
tubing having a textured surface.

10   By "foreign DNA sequence" is meant a DNA sequence
which differs from that of the wild type genomic DNA of
the organism and may be extra-chromosomal, integrated
into the chromosome, or the result of a mutation in the
genomic DNA sequence.

15   By "substantially post-mitotic cells" is meant an
organoid in which at least 50% of the cells containing a
foreign DNA sequence are non-proliferative. Preferably,
organoids including substantially post-mitotic cells are
those in which at least 80% of the cells containing a
20 foreign DNA sequence are non-proliferative. More
preferably, organoids including substantially post-
mitotic cells are those in which at least 90% of the
cells containing a foreign DNA sequence are non-
proliferative. Most preferably, organoids including
25 substantially post-mitotic cells are those in which 99%
of the cells containing a foreign DNA sequence are non-
proliferative. Cells of an organoid retaining
proliferative capacity may include cells of any of the
types included in the tissue. For example, in skeletal
30 muscle organoids, the proliferative cells may include
muscle stem cells (i.e., satellite cells) and
fibroblasts.

The invention provides a number of advantages.
For example, implantation of an organized tissue produced
35 in vitro provides quantifiable, reproducible, and

- 11 -

localized delivery of bioactive compounds to an organism.
Prior to implantation, the production of bioactive
compounds by the organized tissue may be measured and
quantified per unit time, per unit mass, or relative to
5   any other physiologically-relevant parameter. In
addition, the capability of an organized tissue to
sustain production of bioactive compounds can be assessed
by culturing for extended periods and assaying of
compound production with time.

10   Moreover, because the organized tissue is
implanted at a defined anatomical location as a discrete
collection of cells, it may be distinguished from host
tissues, removed post-implantation from the organism, and
reimplanted into the organism at the same or a different
15   location at the time of removal or following an interim
period of culturing in vitro. This feature facilitates
transient or localized delivery of the bioactive
compound. Restriction of the cells producing bioactive
compounds to particular anatomical sites also enhances
20   the controlled delivery of bioactive compounds,
especially where the organized tissue functions as a
paracrine organ. The efficiency of delivery of a
bioactive compound (i.e., the amount of the bioactive
compound delivered to obtain a desired serum
25   concentration) is also enhanced as compared to direct
subcutaneous injection. Likewise, the efficiency of
implanting post-mitotic cells containing a foreign DNA
sequence into an organism (i.e., the number of cells in a
post-mitotic state as a percentage of the initial number
30   of cells containing the foreign DNA sequence) is enhanced
by organoid implantation as compared to the implantation
of individual mitotic cells. For example, skeletal
muscle organoids produced in vitro include post-mitotic
myofibers representing greater than 70% of the initial
35   myoblasts containing a foreign DNA sequence, whereas

- 12 -

direct implantation of the myoblasts results in post-
mitotic myofibers representing less than 1% of the
initial cells.

In addition, because substantially all of the
5 implanted cells are fully differentiated, migration of
these cells to other anatomical sites is reduced.
Moreover, implantation of post-mitotic, non-migratory
myofibers containing a foreign DNA reduces the
possibility of cell transformation and tumor formation.
10 The implantation of an organized tissue may even enhance
the functional and structural characteristics of the host
tissue.

Furthermore, because the method of producing a
tissue having an *in vivo*-like gross and cellular
15 morphology may be achieved without the application of
external forces by mechanical devices, the apparatus for
producing such a tissue is readily adaptable to standard
cell and tissue culture systems. The apparatus and
method may also be used to produce bone, cartilage,
20 tendon, and cardiac tissues as these tissues include cell
types which organize in response to external forces. In
addition, the apparatus includes widely available, easily
assembled and relatively inexpensive components.

Other advantages and features of the invention
25 will be apparent from the detailed description, and from
the claims.

Brief Description of the Drawings

FIG. 1 is a diagram of a vessel for growing
skeletal muscle tissue which will have an *in vivo*-like
30 gross and cellular morphology.

FIG. 2 is a flow chart of the process of skeletal
muscle growth and regeneration.

FIG. 3 is a photograph of skeletal muscle
organoids formed *in vitro* from rhGH-secreting C2C12
35 cells.

- 13 -

FIG. 4 is a micrograph of a section of a skeletal muscle organoid grown *in vitro* from rhGH-secreting C2C12 cells which has been stained for sarcomeric tropomyosin.

FIG. 5 is a micrograph of a section of a skeletal
5   muscle organoid grown *in vitro* from rhGH-secreting C2C12 cells which has been stained for sarcomeric tropomyosin.

FIG. 6 is a flow chart comparing myoblast and myofiber gene therapy methods.

FIG. 7A-7F are graphs of rhGH serum levels in mice
10 following skeletal muscle organoid implantation.

FIG. 8A-8B are graphs of the effects of cytosine arabinoside on rhGH-secreting C2C12 proliferating myoblasts and post-mitotic myofibers.

FIG. 9A-9C are photographs of a skeletal muscle
15 organoid grown *in vitro* from rhGH-secreting C2C12 cells, implanted *in vivo*, and subsequently removed and further cultured *in vitro*.

Fig. 10A-10C are Northern blots of rhBMP-6 mRNA levels in C2C12 cells retrovirally-transduced with a
20 rhBMP-6 gene.

Fig. 11 is a graph of alkaline phosphatase activity in controls and C2C12 cells retrovirally-transduced with a rhBMP-6 gene.

Fig. 12A and 12B are micrographs of C2C12 cells
25 retrovirally-transduced with a rhBMP-6 gene which have been stained for sarcomeric tropomyosin.

Detailed Description
I.   *In Vitro* Production of Tissues Having
     In Vivo-like Gross and Cellular Morphology
30

Organized tissues having *in vivo*-like gross and cellular morphology may be produced *in vitro* from the individual cells of a tissue of interest. As a first step in this process, disaggregated or partially

- 14 -

disaggregated cells are mixed with a solution of
extracellular matrix components to create a suspension.
This suspension is then placed in a vessel having a three
dimensional geometry which approximates the *in vivo* gross
5 morphology of the tissue and includes tissue attachment
surfaces coupled to the vessel. The cells and
extracellular matrix components are then allowed to
coalesce or gel within the vessel, and the vessel is
placed within a culture chamber and surrounded with media
10 under conditions in which the cells are allowed to form
an organized tissue connected to the attachment surfaces.

Although this method is compatible with the *in
vitro* production of a wide variety of tissues, it is
particularly suitable for tissues in which at least a
15 subset of the individual cells are exposed to and
impacted by mechanical forces during tissue development,
remodeling or normal physiologic function. Examples of
such tissues include muscle, bone, skin, nerve, tendon,
cartilage, connective tissue, endothelial tissue,
20 epithelial tissue, and lung. More specific examples
include skeletal, cardiac, and smooth muscle, stratified
or lamellar bone, and hyaline cartilage. Where the
tissue includes a plurality of cell types, the different
types of cells may be obtained from the same or different
25 organisms, the same or different donors, and the same or
different tissues. Moreover, the cells may be primary
cells or immortalized cells. Furthermore, all or some of
the cells of the tissue may contain a foreign DNA
sequence which mediates the production of a bioactive
30 compound (as described herein).

The composition of the solution of extracellular
matrix components will vary according to the tissue
produced. Representative extracellular matrix components
include, but are not limited to, collagen, laminin,
35 fibronectin, vitronectin, elastin, glycosaminoglycans,

- 15 -

proteoglycans, and combinations of some or all of these
components (e.g., Matrigel*, Collaborative Research,
Catalog No. 40234). In tissues containing cell types
which are responsive to mechanical forces, the solution

5 of extracellular matrix components preferably gels or
coalesces such that the cells are exposed to forces
associated with the internal tension in the gel.

Culture conditions will also vary according to the
tissue produced. Methods for culturing cells are well

10 known in the art and are described, for example, in
Animal Cell Culture: A Practical Approach, (R.I.
Fveshney, ed. IRL Press, 1936). In general, the vessel
containing a coalesced suspension of cells and
extracellular matrix components is placed in a standard

15 culture chamber (e.g., wells, dishes, or the like), and
the chamber is then filled with culture medium until the
vessel is submerged. The composition of the culture
medium is varied, for example, according to the tissue
produced, the necessity of controlling the proliferation

20 or differentiation of some or all of the cells in the
tissue, the length of the culture period and the
requirement for particular constituents to mediate the
production of a particular bioactive compound. The
culture vessel may be constructed from a variety of

25 materials in a variety of shapes as described below.

An apparatus for producing a tissue in vitro
having an in vivo-like gross and cellular morphology
includes a vessel having a three dimensional geometry
which approximates the in vivo gross morphology of the

30 tissue. The apparatus also includes tissue attachment
surfaces coupled to the vessel. Such a vessel may be
constructed from a variety of materials which are
compatible with the culturing of cells and tissues (e.g.,
capable of being sterilized and compatible with a

35 particular solution of extracellular matrix components)

- 16 -

and which are formable into three dimensional shapes
approximating the *in vivo* gross morphology of a tissue of
interest. The tissue attachment surfaces (e.g.,
stainless steel mesh, VELCRO", or the like) are coupled to
5   the vessel and positioned such that as the tissue forms
in vitro the cells may adhere to and align between the
attachment surfaces. The tissue attachment surfaces may
be constructed from a variety of materials which are
compatible with the culturing of cells and tissues (e.g.,
10 capable of being sterilized, or having an appropriate
surface charge, texture, or coating for cell adherence).

The tissue attachment surfaces may be coupled in a
variety of ways to an interior or exterior surface of the
vessel. Alternatively, the tissue attachment surfaces
15 may be coupled to the culture chamber such that they are
positioned adjacent the vessel and accessible by the
cells during tissue formation. In addition to serving as
points of adherence, in certain tissue types (e.g.,
muscle), the attachment surfaces allow for the
20 development of tension by the tissue between opposing
attachment surfaces. Moreover, where it is desirable to
maintain this tension *in vivo*, the tissue attachment
surfaces may be implanted into an organism along with the
tissue (see further discussion in Section II.).

25   One vessel according to the invention is shown in
Fig. 1. This vessel 1, which is suitable for the *in
vitro* production of a skeletal muscle organoid 3, has a
substantially semi-cylindrical shape and tissue
attachment surfaces 2 coupled to an interior surface of
30 the vessel.

A.   *In Vitro* Production of a Skeletal Muscle Organoid
        having *In Vivo-Like Gross and Cellular Morphology*
        Using an apparatus and method as generally
described above, a skeletal muscle organoid having an *in*

vivo-like gross and cellular morphology was produced *in vitro*. An overview of the stages of skeletal muscle growth and regeneration is shown in Fig. 2. As shown, during skeletal muscle development embryonic myoblasts
5 proliferate, differentiate, and then fuse to form multi-nucleated myofibers. Although the myofibers are non-proliferative, a population of muscle stem cells (i.e., satellite cells), derived from the embryonic myoblast precursor cells, retain their proliferative capacity and
10 serve as a source of myoblasts for muscle regeneration in the adult organism. Therefore, either embryonic myoblasts or adult skeletal muscle stem cells may serve as one of the types of precursor cells for *in vitro* production of a skeletal muscle organoid.
15   To produce skeletal muscle cells capable of secreting a bioactive compound, primary rat or avian cells or immortalized murine cells secreting recombinant human growth hormone, were suspended in a solution of collagen and Matrigel™ which was maintained at 4°C to
20 prevent gelling. The cell suspension was then placed in a semi-cylindrical vessel with tissue attachment surfaces coupled to an interior surface at each end of the vessel. The vessel was positioned in the bottom of a standard cell culture chamber. Following two to four hours of
25 incubation at 37°C, the gelled cell suspension was covered with fresh culture medium (renewed at 24 to 72 hour intervals) and the chamber containing the suspended cells was maintained in a humidified 5% CO_2 incubator at 37°C throughout the experiment.
30   Between the second and sixth day of culture, the cells were found to be organized to the extent that they spontaneously detached from the vessel. At this stage, the cells were suspended in culture medium while coupled under tension between tissue attachment surfaces
35 positioned at either end of the culture vessel. During

- 18 -

the subsequent ten to fourteen days, the cells formed an
organoid containing skeletal myofibers aligned parallel
to each other in three dimensions. The alignment of the
myofibers and the gross and cellular morphology of the
5 organoid were similar to that of in vivo skeletal muscle.

To carry out the above method, an apparatus for
organoid formation was constructed from silastic tubing
and either VELCRO™ or metal screens as follows. A
section of silastic tubing (approximately 5 mm I.D., 8 mm
10 O.D., and 30 mm length) was split in half with a razor
blade and sealed at each end with silicone rubber
caulking. Strips of VELCRO™ (loop or hook side, 3 mm
wide by 4 mm long) or L-shaped strips of stainless steel
screen (3 mm wide by 4 mm long by 4 mm high) were then
15 attached with silicone rubber caulking to the interior
surface of the split tubing near the sealed ends. The
apparatus was thoroughly rinsed with distilled/deionized
water and subjected to gas sterilization.

Skeletal muscle organoids were produced in vitro
20 from a C2C12 mouse skeletal muscle myoblast cell line
stably co-transfected with recombinant human growth
hormone-expressing and β-galactosidase-expressing (β-gal)
constructs. Dhawan et al., Science 254:1509-1512, 1991.
Cells were plated in the vessel at a density of 1-4 X 10⁶
25 cells per vessel in 400 μl of a solution containing
extracellular matrix components. The suspension of cells
and extracellular matrix components was achieved by the
following method. The solution includes 1 part Matrigel™
(Collaborative Research, Catalog No. 40234) and 6 parts
30 of a 1.6 mg/ml solution of rat tail Type I collagen
(Collaborative Research, Catalog No. 40236). The
Matrigel™ was defrosted slowly on ice and kept chilled
until use. The collagen solution was prepared just prior
to cell plating by adding to lyophilized collagen, growth
35 medium (see constituents below), and 0.1N NaOH in volumes

- 19 -

equivalent to 90% and 10%, respectively, of the volume
required to obtain a final concentration of 1.6 mg/ml and
a pH of 7.0-7.3. The collagen, sodium hydroxide and
growth medium were maintained on ice prior to and after
5 mixing by inversion.

Freshly centrifuged cells were suspended in the
collagen solution by trituration with a chilled sterile
pipet. Matrigel™ was subsequently added with a chilled
pipet and the suspension was once again mixed by
10 trituration. The suspension of cells and extracellular
matrix components was maintained on ice until it was
plated in the vessel using chilled pipet tips. The
solution was pipetted and spread along the length of the
vessel, taking care to integrate the solution into the
15 tissue attachment surfaces. The culture chamber
containing the vessel was then placed in a standard cell
culture incubator, taking care not to shake or disturb
the suspension. The suspension was allowed to gel, and
after 2 hours the culture chamber was filled with growth
20 medium such that the vessel was submerged.

For a period of three days the cells were
maintained on growth medium containing DMEM-high glucose
(GIBCO-BRL), 5% fetal calf serum (Hyclone Laboratories),
and 1% penicillin/streptomycin solution (final
25 concentration 100 units/ml and 0.1 μg/ml, respectively).
On the fourth day of culture, the cells were switched to
fusion medium containing DMEM-high glucose, 2% horse
serum (Hyclone Laboratories), and 100 units/ml penicillin
for a period of 4 days. On the eighth day of culture,
30 the cells were switched to maintenance medium containing
DMEM-high glucose, 10% horse serum, 5% fetal calf serum,
and 100 units/ml penicillin for the remainder of the
experiment. Before the organoids were ready for
implantation, some were cultured in maintenance media
35 containing 1 μg/ml of cytosine arabinoside for the final

four to eight days. Treatment with cytosine arabinoside eliminated proliferating cells and produced organoids including substantially post-mitotic cells.

The cell-extracellular matrix gel (cell-gel)
5   formed in vitro from these stably transfected C2C12 cells 48 hours after plating are shown in Fig. 3. In the upper half of the figure the cell-gel has detached from one of the tissue attachment surfaces. The resultant contraction demonstrates the tension developed in the gel
10 between the tissue attachment surfaces. Figs. 4 and 5 demonstrate the presence of a muscle-specific contractile protein (i.e., brown staining following incubation with an antibody to sarcomeric tropomyosin), in parallel arrays of highly organized and longitudinally oriented
15 myofibers in mammalian skeletal muscle organoids following three weeks of culturing in the apparatus shown in Fig. 1. Moreover, Fig. 9B shows the retention of myofiber organization following organoid implantation.

II.   Delivery of Bioactive Compounds
20   Bioactive compounds may be delivered to an organism by growing individual cells in vitro under conditions that result in the formation of an organized tissue producing the bioactive compound and subsequently implanting the organized tissue into the organism (see
25 Section I. for detailed description of organized tissue production). Production of the bioactive compound by the organized tissue is mediated by a foreign DNA sequence present in at least a subset of the cells which make up the implanted tissue.
30   A variety of bioactive compounds may be delivered by this method, and they may function through intracellular (i.e., within the cells of the organized tissue or organoid), endocrine, autocrine, or paracrine mechanisms. Moreover, the organoid may deliver multiple

bioactive compounds either simultaneously or sequentially
(e.g., one bioactive compound mediates the delivery of
another). Liberation of the bioactive compound from the
cells of the organoid may occur by either passive or
5 active processes (e.g., diffusion or secretion).

For example, the bioactive compound may be a
hormone, growth factor, or the like which is produced and
liberated by the cells of the organoid to act locally or
systemically on host tissues. Alternatively, the
10 bioactive compound may function within the cells or on
the surface of the cells of the organoid to enhance the
uptake or metabolism of compounds from the host tissue or
circulation (e.g., lactic acid, low density lipoprotein).
Where the organoid serves as a functional and structural
15 adjunct to the host tissue, delivery of growth factors by
autocrine or paracrine mechanisms may enhance the
integration of the organoid into host tissues.
Similarly, where multiple bioactive compounds are
produced by the organoid, autocrine delivery of one of
20 the bioactive compounds may be used to regulate the
production of one or more of the other bioactive
compounds.

The organoid may be implanted by standard
laboratory or surgical techniques at a desired anatomical
25 location within the organism. For example, the organoid
may be implanted in the same or a different tissue from
the tissue of origin of at least one of the individual
cells. The location of implantation depends, in part,
upon the method of delivery and the identity of the
30 particular bioactive compound to be delivered. For
example, an organoid acting as an endocrine organ may be
implanted in or adjacent a highly vascularized host
tissue. Alternatively, an organoid acting as a paracrine
organ is preferably implanted in or adjacent to the host

- 22 -

tissue to which the bioactive compound is to be
delivered.

The organoid may be implanted by attachment to a
host tissue or as a free floating tissue. In addition,
5 attached organoids may be implanted with or without the
tissue attachment surfaces used for *in vitro* tissue
formation. Tissues responsive to mechanical forces are
preferably implanted by attaching directly to the host
tissue or by implanting the organoid coupled to the
10 attachment surfaces so that the organoid is exposed to
mechanical forces *in vivo*. For example, skeletal muscle
organoids are preferably implanted by attachment to the
host tissue under tension along a longitudinal axis of
the organoid. Moreover, the organoids may be permanently
15 or temporarily implanted. Permanent implantation may be
preferred, for example, where the organoid produces a
bioactive compound which corrects a systemic metabolic
error (e.g., delivery of insulin to treat diabetes),
whereas temporary implantation may be preferred where
20 only transient delivery of a bioactive compound is
desired (e.g., delivery of a growth factor to enhance
wound healing). Furthermore, because organoids may be
implanted, removed, and maintained *in vitro* (see Fig. 9A
and discussion below), bioactive compounds may be
25 delivered intermittently to the same or a different
location in the organism. For example, a skeletal muscle
organoid produced from the cells of a human patient
(e.g., an autograft) may be implanted at a first
anatomical location for a defined period and subsequently
30 implanted at a second location at or after the time of
removal.

At least some of the cells of the organoid contain
a foreign DNA sequence. The foreign DNA sequence may be
extra-chromosomal, integrated into the genomic DNA of the
35 organoid cell, or may result from a mutation in the

- 23 -

genomic DNA of the organoid cell. In addition, the cells
of the organoid may contain multiple foreign DNA
sequences. Moreover, the different cells of the organoid
may contain different foreign DNA sequences. For

5   example, in one embodiment, a skeletal muscle organoid
may include myofibers containing a first foreign DNA
sequence and fibroblasts containing a second foreign DNA
sequence. Alternatively, the skeletal muscle organoid
could include myoblasts from different cell lines, each

10 cell line expressing a foreign DNA sequence encoding a
different bioactive compound. These "mosaic" organoids
allow the combined and/or synergistic effects of
particular bioactive compounds to be exploited. For
example, myoblasts expressing growth hormone may be

15 combined with myoblasts expressing an insulin-like growth
factor to produce organoids useful in stimulating muscle
growth/regeneration. Similarly, myoblasts expressing a
bone morphogenetic protein may be combined with myoblasts
expressing a parathyroid hormone to produce organoids

20 useful in stimulating bone and cartilage
growth/regeneration.

        In a preferred embodiment, the foreign DNA
sequence encodes a protein which is the bioactive
compound. The protein is produced by the cells and

25 liberated from the organoid. Alternatively, the DNA
sequence may encode an enzyme which mediates the
production of a bioactive compound or a cell surface
protein which enhances the uptake and metabolism of
compounds from the host tissue or circulation (e.g.,

30 lactic acid or low density lipoproteins). The DNA
sequence may also encode a DNA binding protein which
regulates the transcription of the sequence encoding a
bioactive compound or an anti-sense RNA which mediates
translation of the mRNA for the bioactive compound. The

35 DNA sequence may also bind trans-acting factors such that

- 24 -

the transcription of the sequence (i.e., foreign or
native) encoding the bioactive compound is enhanced
(e.g., by disinhibition). Furthermore, the foreign DNA
sequence may be a cis-acting control element such as a
5   promoter or an enhancer coupled to a native or foreign
coding sequence for the bioactive compound or for an
enzyme which mediates the production of the bioactive
compound.

A.   Delivery of Human Growth Hormone to Mice by
10   Implanting Skeletal Muscle Organoids
        Figure 6 shows an overview and comparison of
myoblast and myofiber gene therapy. Both methods
generally involve isolating myoblasts from a patient in
need of gene therapy, inserting into the myoblasts a DNA
15 sequence encoding a bioactive compound, and expanding the
myoblast cell population by in vitro culturing.   In
contrast to myoblast gene therapy, the myoblasts used in
myofiber gene therapy are further cultured in vitro under
conditions which result in the formation of an organoid
20 having in vivo-like gross and cellular morphology. The
organoid is subsequently implanted into the patient to
deliver the bioactive compound.
        To carry out the delivery of a bioactive compound
to an organism, skeletal muscle organoids were formed in
25 vitro, as described above, from C2C12 mouse skeletal
muscle myoblasts stably co-transfected with recombinant
human growth hormone-expressing and β-galactosidase-
expressing constructs. Prior to implantation, in vitro
production of recombinant human growth hormone ("rhGH")
30 was measured by radioimmunoassay according to the
manufacturer's instructions (Nichols Institute
Diagnostics, San Juan Capistrano, CA). Between three and
twenty-four days of culture, the mean rhGH production

- 25 -

ranged between 1.0 and 3.5 μg/day/organoid (see Table 1).

TABLE 1

IN VITRO PREIMPLANT SUMMARY

Experiment	Date	Initial Cell # per Organoid (x 10^4)	Age of Organoid (Days)	Mean rhGH (μg/day/ organoid) (N=)	Treatment of Organoids
IMPLANT 1	8/24	6	3 7	1.9 (2) 3.5 (2)	none
IMPLANT 2	9/21	-	-	-	-
IMPLANT 3	10/5	4	7 12	1.7 - 2.8 (7) 1.9 - 2.5 (6)	none
IMPLANT 4	10/20	2	??	2.2 - 2.6 (5)	none
IMPLANT 5	10/25	2	12 12	2.9 (12) 2.0 (4)	no cytosine arabinoside ("araC") 1 ug/ml araC for 4 days
IMPLANT 6	11/8	3	19	1.9 (6) 1.0 (6)	no araC 1 ug/ml araC for 5 days
IMPLANT 7	11/9	3 (non-rhGH secreting)	2	0 (3)	control experiment
IMPLANT 8	11/3	2	14-20	1.5 to 2.2 (6) 1.2 to 1.6 (6)	no araC 1 ug/ml araC for 5 days
IMPLANT 9	11/30	1 - 2	24	1.7 to 2.4 (8)	1 ug/ml araC for 8 days
IMPLANT 10	12/5	1.5 - 2.0	20	2.1 to 2.9 (14)	1 ug/ml araC for 4 days

The organoids were implanted into adult C3HeB/FeJ mice (i.e., syngeneic to C2C12 cells) by the following method. Mice were weighed to determine dosages of cyclosporine and anesthetic. One hour prior to the surgical implantation of the organoid, each mouse was given an injection of 60 mg/kg of cyclosporine A. Each mouse was then selected in turn and anesthetized by intramuscular injection of 55 mg/kg Ketamine, 1 mg/kg Promazine, and 5 mg/kg Xylazine. The site of implantation was then depilated with Nair™ or by

- 26 -

shaving, and prepped for aseptic surgery.   For organoids
implanted subcutaneously, a four to six centimeter long
incision was made along the back, the organoid was
implanted in either a free floating state or fixed under
5 tension (e.g., attached to the tissue attachment
surfaces), and the incision was closed with four to six
sutures of 4.0-black silk.

   For organoids implanted intramuscularly, a 15 to
30 millimeter incision was made parallel to the anterior
10 tibialis muscle (e.g., anteriolateral aspect of the lower
hind limb) to provide access to the muscle sheath.   The
anterior tibialis was gently split with forceps from
tendon to tendon parallel to the muscle belly, thus
providing a cavity for insertion of the organoid.   The
15 organoid was carefully removed from the vessel by prying
the ends off the tissue attachment surfaces with sterile
forceps and inserting it, under resting tension, in the
implantation site.   The incision was closed as described
above.   Mice were then followed post-surgically for
20 distress and upon regaining consciousness were returned
to an animal care facility.   Cyclosporine injections are
repeated daily for the duration of the experiment.   The
experimental protocol for the implantation of skeletal
muscle organoids is summarized in Table 2 below.

- 27 -

TABLE 2

IN VIVO PROTOCOL SUMMARY

Experiment	Date	Site of Implant	# of Surviving Animals	rhGH Producers (# and method of implant)
IMPLANT 1	8/24	intramuscular free-floating	2 of 2	0 (1 free)
IMPLANT 2	9/21	controls only - cyclosporine dose-response	6 of 6	no organoids implanted
IMPLANT 3	10/5	subcutaneous free-floating	3 of 4	1 (3C - 2 free)
IMPLANT 4	10/20	subcutaneous fixed under tension	2 of 3	2 (2D - 2 fixed) (3D- 1 fixed/1 free)
IMPLANT 5	10/25	subcutaneous fixed under tension	1 of 2	1 (1E - 3 fixed)
IMPLANT 6	11/8	subcutaneous fixed under tension	4 of 7	3 (6A, 6D, 6E - 1 fixed) (6G - no organoid)
IMPLANT 7	11/9	subcutaneous fixed under tension	2 of 3	0 (7A and 7C - 1 fixed, non-rhGH secreting organoid)
IMPLANT 8	11/13	subcutaneous fixed under tension	3 of 8	4 (8C, 8D, 8F and 8G - 1 fixed)
IMPLANT 9	11/30	subcutaneous fixed under tension or free-floating	7 of 7	5 (9A, 9B, 9C, 9D and 9F - 1 fixed) 1 (9E - 1 free) (9G - no organoid)
IMPLANT 10	12/2	subcutaneous fixed under tension	7 of 11	7 (10A, 10B, 10C, 10D, 10F, 10G, and 10J - 1 fixed)

Blood was collected every one to seven days by
tail bleeding from the mice. Sera concentrations of rhGH
were measured by radioimmunoassay according to the
manufacturer's instructions (Nichols Institute
Diagnostics, San Juan Capistrano, CA).

As shown in Figs. 7A-7F, rhGH was detected in the
blood of animals receiving rhGH organoid implants, but
not in controls (6G, 7A, 7C, and 9G) for up to thirty-
three days post-implantation. Serum concentrations were

- 28 -

elevated as high as approximately 5.5 to 9 ng/ml in
animals receiving multiple implants of rhGH producing
organoids (1E, 2D), whereas serum from animals receiving
no implant (6G, 9G) or implants of non-rhGH secreting
5 organoids (7A and 7C) contained no detectable rhGH. In
addition, animals receiving organoids treated in vitro
with cytosine arabinoside prior to implantation (1E, 6E,
8D, 8F, 8G, 9A through 9F, and 10A through 10J)
demonstrated serum rhGH levels comparable to those of
10 animals receiving implants which were not treated in
vitro with cytosine arabinoside prior to implantation
(i.e., 2D, 3C, 3D, 6A, 6D, and 8C). Under the conditions
used in this study, cytosine arabinoside treatment kills
greater than 99% of proliferating C2C12 myoblasts while
15 having only a minor effect on myofiber metabolism and
rhGH secretion (Fig. 8). Moreover, Fig. 9C shows that
the rhGH gene and the β-galactosidase gene are only
expressed in post-mitotic myofibers. These results
demonstrate that organoids including substantially post-
20 mitotic cells can deliver therapeutic levels of a
bioactive compound for up to thirty-three days post-
implantation.

        It is noteworthy that within forty-eight hours
following the removal of implants (i.e., 8D, 8G and 9F),
25 rhGH was undetectable in the sera of animals previously
having serum concentrations as high as 2.6 ng/ml. These
data demonstrate the reversibility of delivering
bioactive compounds by this method. In addition,
organoids removed from animals may be re-incubated in
30 vitro (see e.g., Fig. 9A). For example, the two organoids
implanted into animal 3D produced 188 ng/day of rhGH in
vitro post-implantation. These data suggest the
feasibility of removing organoids and subsequently
reimplanting them such that bioactive compounds may be
35 delivered during multiple treatment periods separated in

- 29 -

time. Moreover, the data suggest the feasibility of
transplanting sequentially, at different sites within the
same organism, organoids functioning as paracrine organs.

      The rhGH production of 188 ng/day *in vitro* by
5 organoids from animal 3D and the *in vivo* serum levels of
1.0 ng/ml on day twenty-four (i.e., just prior to
removal) suggest a 188-fold difference between organoid
production and steady state circulating levels of rhGH in
the animal. These results compare favorably to the 500-
10 fold difference between rhGH concentrations delivered by
direct subcutaneous injection and steady state
circulating levels, (Yang et al. *Circulation* 92:262-267,
1995 (1000 μg/day rhGH by direct subcutaneous injection
produced 2 μg/ml serum concentrations in rats). It is
15 also noteworthy that the organoid maintained *in vivo*
under tension produced approximately 144 ng/ml when
placed *in vitro* on removal from the animal, while the
free floating organoid produced only 40 ng/ml when placed
in vitro on removal from the animal. In addition, an
20 organoid implanted under no tension (9E) was a poorer
producer of rhGH *in vivo* than those placed under tension
(9A, 9B, 9C). These results suggest that maintaining
organoids under tension enhances the production and
delivery of bioactive compounds.

25 B.   Delivery of Bone Morphogenetic Protein to an
          Organism by Implanting Skeletal Muscle Organoids

          1.   Transduction and Selection of C2C12 Myoblasts
                Expressing rhBMP-6
          ψ2 packaging cells producing high titers (> 1 x
30 10^7 pfu) of retrovirus containing the pLX(rhBMP-6)SN
expression vector were provided by Dr. Vladimir Drozdoff,
Department of Medicine, Vanderbilt University. Myoblast

- 30 -

cell cultures, 50% confluent in T-75 flasks, were
incubated for eight hours in 20 ml of conditioned media
from the high viral titer packaging cells. The media was
supplemented with 4 μg/ml of polybreen. After eight
5   hours, the cells were placed in DMEM + 10% fetal calf
serum containing 2 μg/ml of polybreen, and cultured for
an additional 48-72 hr, or until the cells had undergone
one or two additional divisions. The transduced cells
were then harvested, counted, and plated out as single
10   cell clones in four 12-well plates. The single cell
clones were selected by culturing in DMEM + 10% fetal
calf serum containing 400 μg/ml of G418. Single cell
colonies began to appear after 2-3 weeks in culture.
These colonies were first expanded to a single T-25
15   flask, and then expanded to two T-150 flasks which were
grown to 90% confluency. The first flask was harvested
for storage of cells in liquid nitrogen, and the second
flask was processed for total RNA.

Alternatively, myoblasts are transducible by
20   direct incubation with plasmids containing bone
morphogenetic protein genes (e.g., mouse BMP-4, Fang, J.,
et al., *Stimulation of new bone formation by direct
transfer of osteogenic plasmid genes*, Proc. Natl. Acad.
Sci. U.S.A. 93:5753-5758, 1996; human BMP-1, BMP-2A and
25   BMP-3, Wozney, J.M., et al., *Novel regulators of bone
formation: molecular clones and activities*, Science
242:1528-1532, 1988; human BMP-4, Ahrens, M., et al.,
*Expression of human bone morphogenetic proteins-2 or -4
in murine mesenchymal progenitor C3H10T1/2 cells induces
30   differentiation into distinct mesenchymal cell lineages*,
DNA and Cell Biology 32:871-880, 1993). For example,
myoblasts may be successfully transduced by standard
calcium phosphate coprecipitation or lipofection.

Northern blot analysis was performed on the cell
35   clones with 20 μg of total or standard RNA per lane

- 31 -

(Figs. 10A-10C). The blots were hybridized with a cDNA
probe to rhBMP-6 (supplied by Genetics Institute,
Cambridge, MA). Referring to Figs. 10A and 10B, clones
expressing high levels of rhBMP-6 mRNA (e.g., cell line
5 4A1 in lane 13 of Fig. 10B) were expanded and recloned
from single cell colonies. Referring to Fig. 10C,
subclones of cell line 4A1 were rescreened by Northern
blot analysis, and clones 1A1 and 2A2 expressed high
levels of rhBMP-6 mRNA relative to the other clones.
10 Cell colonies retaining high expression of rhBMP-6 were
harvested and banked in liquid nitrogen.

        2.   Expression of Biologically Active BMP-6
        The biological activity of rhBMP-6 in cell
colonies retaining high expression of rhBMP-6 (i.e., C_2-
15 BMP6 cells) was determined by measuring alkaline
phosphate activity (i.e., an osteoblastic marker) in the
cells after 14 days in culture. Normal C2C12 cells
(i.e., non-transduced cells) and C2C12 cells transduced
with the LXSN vector alone (i.e., C_2-LXSN cells) were used
20 as controls.
        Cells were harvested after 14 days as follows.
Wells containing the cells were rinsed with phosphate
buffered saline (0.1m, pH 7.4; PBS) and then typsinized
with five drops per well of 0.05% trypsin/EDTA solution
25 in PBS. The trypsin/EDTA was neutralized with 500 μl
serum-containing media per well, and cells were
transferred to microcentrifuge tubes and centrifuged at
900 rpm for four minutes to pellet the cells. Cell
pellets were resuspended and lysed in 500 μl of TXM
30 buffer (10 mM Tris HCL; 1.0 mM magnesium chloride; 0.02
mM zinc chloride; 0.1% Triton X-100; and 0.02% sodium
azide), and stored at -20°C until assayed or assayed
immediately for alkaline phosphate activity as follows.

- 32 -

One hundred microliters of cell lysate, blank
(buffer minus substrate), or standard (5 mM p-nitrophenol
in buffer) was added to a tube containing 400 µl of
alkaline phosphate assay substrate and buffer (0.1 mg
5 glycine; 2.0 mM magnesium chloride; 2 mg/ml p-nitrophenyl
phosphate) and incubated at 37°C for 30 min. The
reaction was stopped by adding 500 µl of 0.25 N NaOH, and
the optical density at 410 nm was read on a
spectrophotometer. The total cellular protein in each
10 sample was measured with a Bio-Rad™ protein assay
essentially according to the manufacturer's instructions
(Bio-Rad Laboratories, Hercules, CA) and alkaline
phosphatase activities calculated as follows:

$$\text{Total Alkaline Phosphatase Activity for Sample } (\frac{\mu g}{hour}) = \frac{(2 \times \text{Sample Optical Density} \times \text{Dilution Factor})}{(\text{Average of Standard Optical Densities})}$$

$$\text{Alkaline Phosphatase Activity } (\frac{\mu g/hour}{mg \text{ cellular protein}}) = \frac{\text{Total Alkaline Phosphatase Activity for Sample}}{\text{Total Cellular Protein for Sample}}$$

- 33 -

3.   Delivery of BMP-6 by Implanting Skeletal
      Muscle Organoids

The ability of C_2-BMP6 cells to differentiate and
fuse to form skeletal muscle myofibers was analyzed by
5 morphometric analysis and expression of the muscle-
specific protein sarcomeric tropomyosin after six to
fourteen days in culture. Normal C2C12 cells and C_2-LXSN
cells were used as controls.

Normal C2C12 cells, C_2-LXSN cells, and C_2-BMP6
10 cells were cultured separately in T-75 flasks. At 80%
confluence, all cell types were individually subcultured
and plated into four well-plates (i.e., 15-mm diameter
wells pretreated with a collagen spray 1 mg/ml of rat-
tail collagen, type I in 1% acetic acid). The cells were
15 plated at a density of 100,000 cells per well in 750 μl
of growth medium (DMEM-high glucose; 10% calf serum; 10%
fetal calf serum; 100 units/ml penicillin; and 0.1 mg/ml
streptomycin) and incubated in a humidified, 37°C, 5% CO_2
atmosphere.

20   The cells were fed 750 μl warm growth medium per
well every 48 hours (i.e., day 2 and day 4 post-plating).
Five days post-plating when all groups showed ~100%
confluence, the cells were switched to a low serum fusion
medium to promote fusion (DMEM-high glucose; 2% horse
25 serum; 100 units/ml penicillin; 0.1 mg/ml streptomycin).
The cells were fed fusion medium on days six, eight and
ten post-plating. On day 12 post-plating, the cells were
switched to a maintenance medium (DMEM-high glucose; 10%
horse serum; 5% fetal calf serum; 100 units/ml
30 penicillin; and 0.1 mg/ml streptomycin). The experiment
was terminated on day 14.

Plates were fixed for morphometric analysis 6, 8,
12 and 14 days post-plating as follows. Cells were
quickly rinsed twice with Eagle's balanced salt solution

(EBSS), fixed with Histochoice™ for thirty minutes at
room temperature, and incubated twice for ten-minutes in
EBSS. The samples were then stored in fresh EBSS at 4°C
until used for immunohistochemical analysis.

5   From storage, samples were warmed to room
temperature and rinsed with phosphate buffer saline (PBS;
10 mM, pH 7.4). Samples were then incubated with the
primary antibody, anti-sarcomeric tropomyosin (1:100
dilution) in 0.5% Tween 20/PBS for thirty minutes at room
10   temperature, followed by PBS rinsing. Secondary antibody
and avidin biotinylated enzyme steps were performed
essentially according to the Vectastain® Elite ABC Kit
protocol. Samples were then developed with
diaminobenzidine tetrahydrochloride (DAB) reagent to
15   produce a brown precipitate, and then lightly
counterstained with hematoxylin.

          Referring to Figs. 12A (Day 8 post-plating) and
12B (Day 14 post-plating), the ability of C_2-BMP6 cells to
differentiate and fuse to form skeletal muscle myofibers
20   is demonstrated by morphometric analysis (i.e., the
presence of longitudinally-oriented multinucleated
fibers) and by the presence of sarcomeric tropomyosin
(i.e., a muscle-specific protein expressed in
differentiated skeletal muscle myofibers but not in
25   undifferentiated, proliferative myoblasts). Because the
expression of a biologically active bone morphogenetic
protein does not impair the ability of skeletal muscle
myoblasts to differentiate and fuse to form skeletal
muscle myofibers, skeletal muscle organoids which express
30   bone morphogenetic proteins are produced as described
above (see Section I), and are used to deliver bone
morphogenetic proteins to an organism also as described
above (see Section II).

          Because bone morphogenetic proteins are
35   extracellular molecules, skeletal muscle organoid

delivery of the protein may be through endocrine,
autocrine, or paracrine mechanisms. In a preferred
embodiment, the organoid may function as a paracrine
organ to deliver a bone morphogenetic protein to
5 chondroblastic or osteoblastic precursor cells. For
example, a skeletal muscle organoid expressing a bone
morphogenetic protein may be implanted adjacent a non-
union fracture to stimulate endochondral bone formation
and repair. Alternatively, a skeletal muscle organoid
10 could be implanted in an organism adjacent skeletal
tissues which are susceptible to degeneration and
fracture consequent to aging (e.g., the hip joint or
spinal column of elderly humans). Similarly, bone
morphogenetic protein expressing organoids may be
15 employed to treat systemic or regional osteoporosis
(e.g., of the spine, femoral neck, and scapular regions
of elderly humans). Skeletal muscle organoids expressing
bone morphogenetic proteins may also function to
accelerate cartilage repair and the healing of segmental
20 defects or bony fusions.

- 36 -

What is claimed is:

1.   A method of delivering a bioactive compound
to an organism, comprising the steps of:

growing a plurality of cells *in vitro* under
5   conditions that allow the formation of an organized
tissue, at least a subset of said cells containing a
foreign DNA sequence which mediates the production of
said bioactive compound; and

implanting said tissue into said organism,
10   whereby said bioactive compound is produced and delivered
to said organism.

2.   The method of claim 1, wherein the step of
growing comprises:

mixing said cells with a solution of
15   extracellular matrix components to create a suspension;

placing said suspension in a vessel having a
three dimensional geometry approximating the *in vivo*
gross morphology of said tissue, said vessel having
attachment surfaces thereon;
20   allowing said suspension to coalesce; and

culturing said coalesced suspension under
conditions in which said cells connect to said attachment
surfaces and form a tissue having an *in vivo*-like gross
and cellular morphology.

25   3.   The method of claim 1, further comprising the
steps of:

removing said tissue from said organism to
terminate delivery of said bioactive compound.

- 37 -

4.   The method of claim 3, further comprising, following said removal step, the step of:

culturing said tissue *in vitro* under conditions which preserve its *in vivo* viability.

5   5.   The method of claim 4, further comprising, following said culturing step, the step of:

reimplanting said tissue into said organism to deliver said bioactive compound to said organism.

6.   The method of claim 1, wherein said tissue is
10   implanted into the tissue of origin of at least one of said cells.

7.   The method of claim 1, wherein said DNA sequence encodes said bioactive compound.

8.   The method of claim 1, wherein said DNA
15   sequence encodes a protein which mediates the production of said bioactive compound.

9.   The method of claim 1, wherein said DNA sequence mediates the production of two bioactive compounds.

20   10.   The method of claim 1, wherein said bioactive compound is a growth factor.

11.   The method of claim 1, wherein said tissue comprises substantially post-mitotic cells.

12.   The method of claim 1, wherein, during said
25   growing step, a force is exerted substantially parallel to a dimension of said tissue.

- 38 -

13.   The method of claim 12, wherein said force is exerted on said individual cells during growth *in vitro* and on said organized tissue during implantation *in vivo*.

14.   The method of claim 1, wherein said tissue
5 comprises skeletal muscle.

15.   The method of claim 1, wherein said tissue comprises myotubes.

16.   The method of claim 1, wherein said organism is a mammal.

10   17.   The method of claim 16, wherein said mammal is a human.

18.   An organized tissue producing a bioactive compound, said tissue produced by the steps of:

mixing a plurality of cells with a solution
15 of extracellular matrix components to create a suspension, at least a subset of said cells containing a foreign DNA sequence which mediates the production of a bioactive compound;

placing said suspension in a vessel having a
20 three dimensional geometry approximating the *in vivo* gross morphology of said tissue, said vessel having attachment surfaces thereon;

allowing said suspension to coalesce; and

culturing said coalesced suspension under
25 conditions in which said cells connect to said attachment surfaces and form a tissue having an *in vivo*-like gross and cellular morphology.

- 39 -

19. An organized tissue producing a bioactive compound, comprising:

a pluraity of cells grown *in vitro* under conditions that allow the formation of an organized
5 tissue; and

a foreign DNA sequence mediating the production of a bioactive compound, said DNA sequence being inserted into at least a subset of said cells.

20. The organized tissue of claim 19, wherein
10 said tissue is skeletal muscle.

21. An *in vitro* method for producing a tissue having an *in vivo*-like gross and cellular morphology, comprising the steps of:

providing precursor cells of said tissue;
15   mixing said cells with a solution of extracellular matrix components to create a suspension;

placing said suspension in a vessel having a three dimensional geometry approximating the *in vivo* gross morphology of said tissue, said vessel having
20 attachment surfaces thereon;

allowing said suspension to coalesce; and

culturing said cells under conditions in which said cells form an organized tissue connected to said attachment surfaces.

25   22. The method of claim 21, wherein the step of providing comprises isolating primary cells of at least one of the cell types comprising said tissue.

23. The method of claim 21, wherein the step of providing comprises utilizing immortalized cells of at
30 least one of the cell types comprising said tissue.

- 40 -

24.   The method of claim 21, wherein the step of providing comprises inserting a foreign DNA sequence into at least one of the cells comprising said tissue.

25.   The method of claim 24, wherein said tissue
5 comprises substantially post-mitotic cells.

26.   The method of claim 21, wherein said coalesced suspension exerts a force on said cells substantially parallel to a dimension of said vessel.

27.   The method of claim 21, wherein said cells
10 are aligned substantially parallel to a dimension of said vessel.

28.   The method of claim 21, wherein said cells comprise skeletal muscle cells.

29.   The method of claim 1, wherein said cells
15 comprise myotubes.

30.   The method of claim 27, wherein said vessel is substantially semi-cylindrical in shape.

31.   The method of claim 30, wherein said attachment surfaces are positioned at opposite ends of
20 said vessel.

32.   The method of claim 21, wherein said tissue produces or mediates the production of a bioactive compound.

33.   An organized tissue produced according to the
25 method of claim 21.

- 41 -

34.   An apparatus for producing a tissue *in vitro*
having an *in vivo*-like gross and cellular morphology,
comprising a vessel having a three dimensional geometry
approximating the *in vivo* morphology of said tissue; and
5   tissue attachment surfaces in said vessel.

35.   The apparatus of claim 34, further comprising
a culture chamber in which said vessel may be submerged.

36.   The apparatus of claim 34, wherein said
vessel is substantially semi-cylindrical in shape.

10   37.   The apparatus of claim 36, wherein said
tissue attachment surfaces are coupled to opposite ends
of said semi-cylindrical vessel.

FIG. 1   Casting Chamber for Tissue Specimen Growth

patent2.vsd

FIG 2. SKELETAL MUSCLE GROWTH AND REGENERATION

EMBRYO

Myoblasts (proliferate)

Fusion

Myofiber (no proliferation)

REGENERATION

Myoblasts (proliferate)

Fusion

Fusion

ADULT

Muscle stem cell
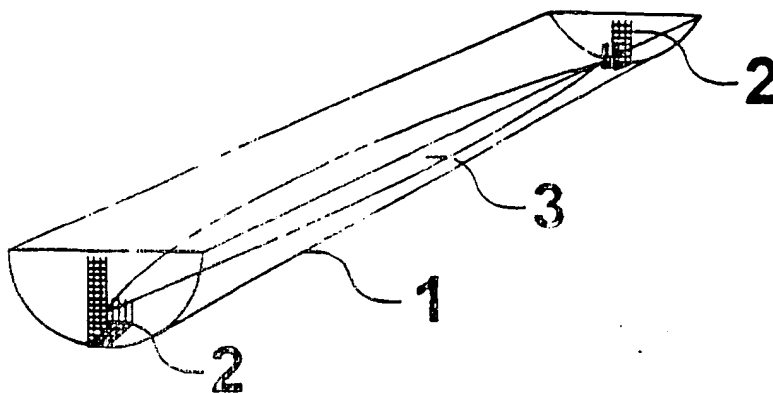
Myofiber (no proliferation)
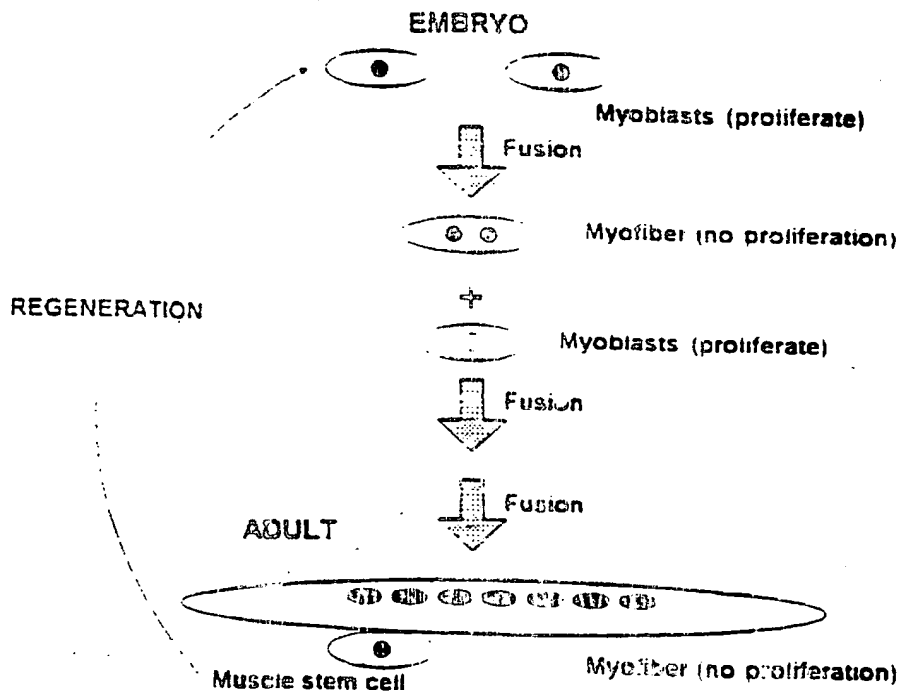
patfig2b.vsd

FIGURE 3. RHGH SECRETING C2C12 CELL-MATRIX
GEL 48 HOURS POSTPLATING. TOP GEL HAS
DETACHED AND CONTRACTED.
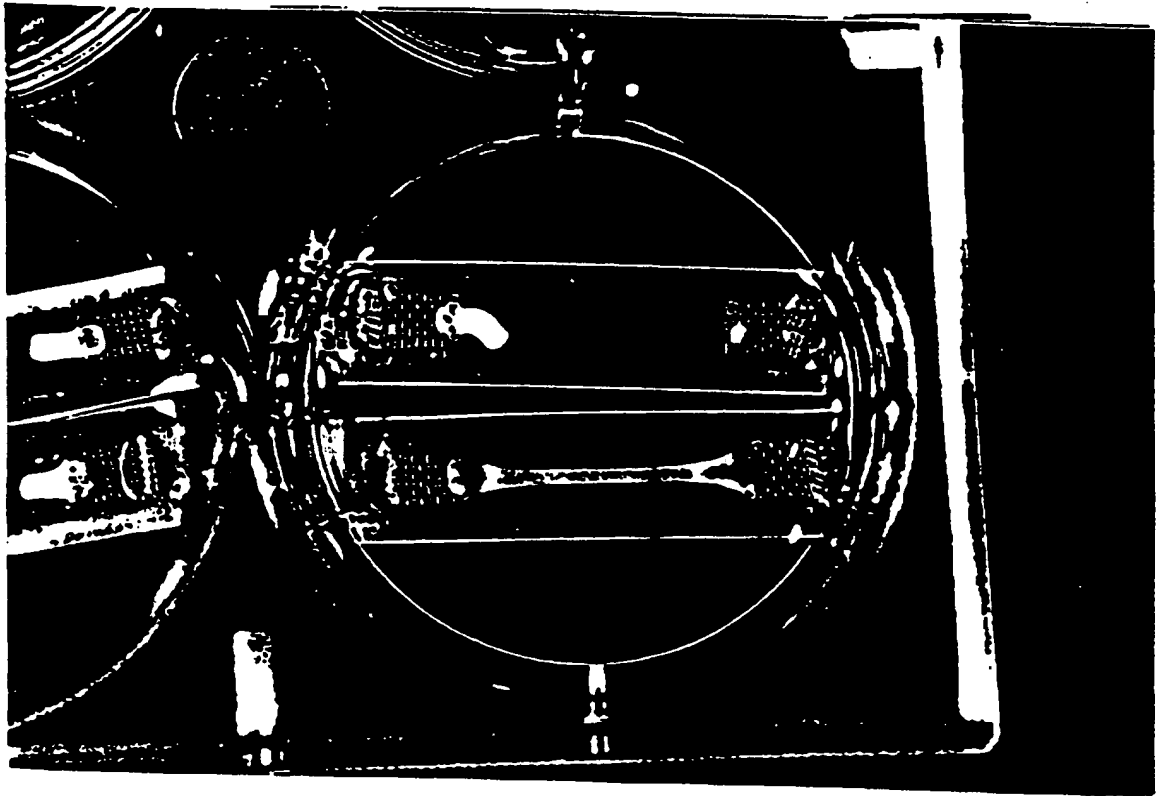
FIGURE 4. Middle section of a 3 weeks old mammalian C2C12 muscle cell organoid stained for sarcomeric tropomyosin, showing longitudinally oriented myofibers (arrows). Magnification is approximately 40X.
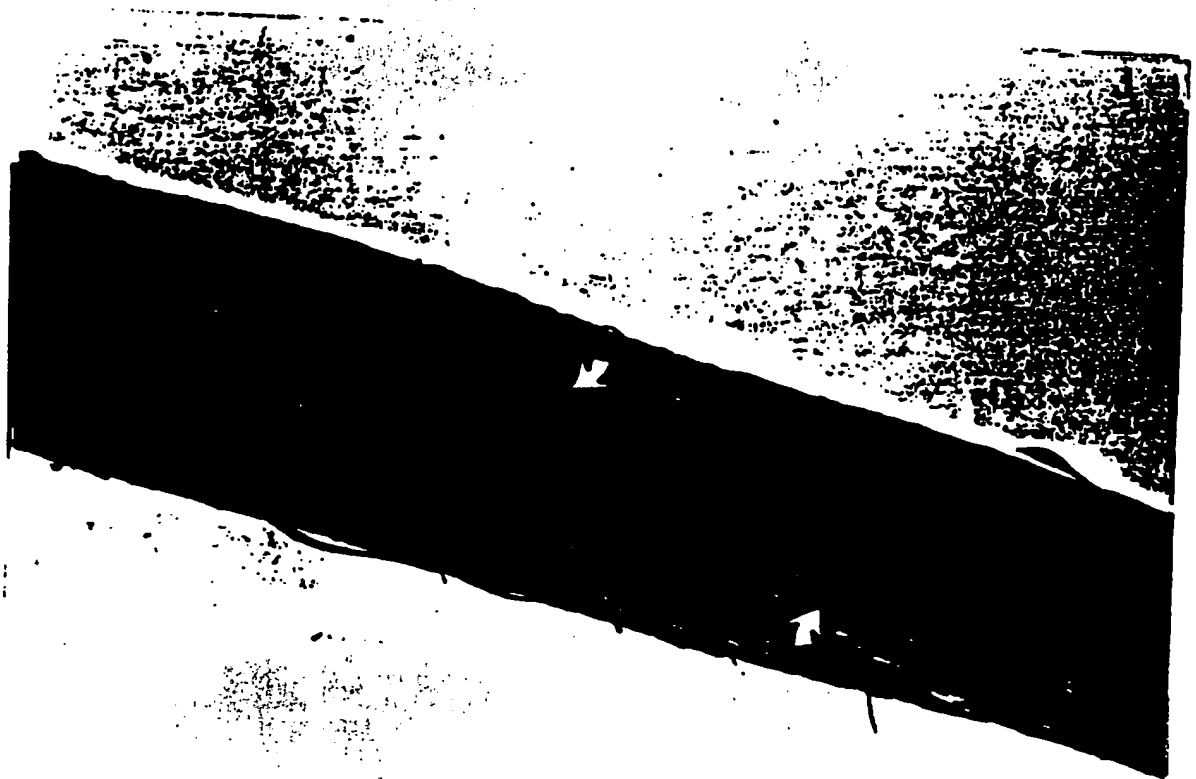
FIGURE 5. Parallel aligned myofibers (arrows) on the surface of a 3 weeks old mammalian C2C12 muscle cell organoid stained for sarcomeric tropomyosin. Magnification is approximately 400x.
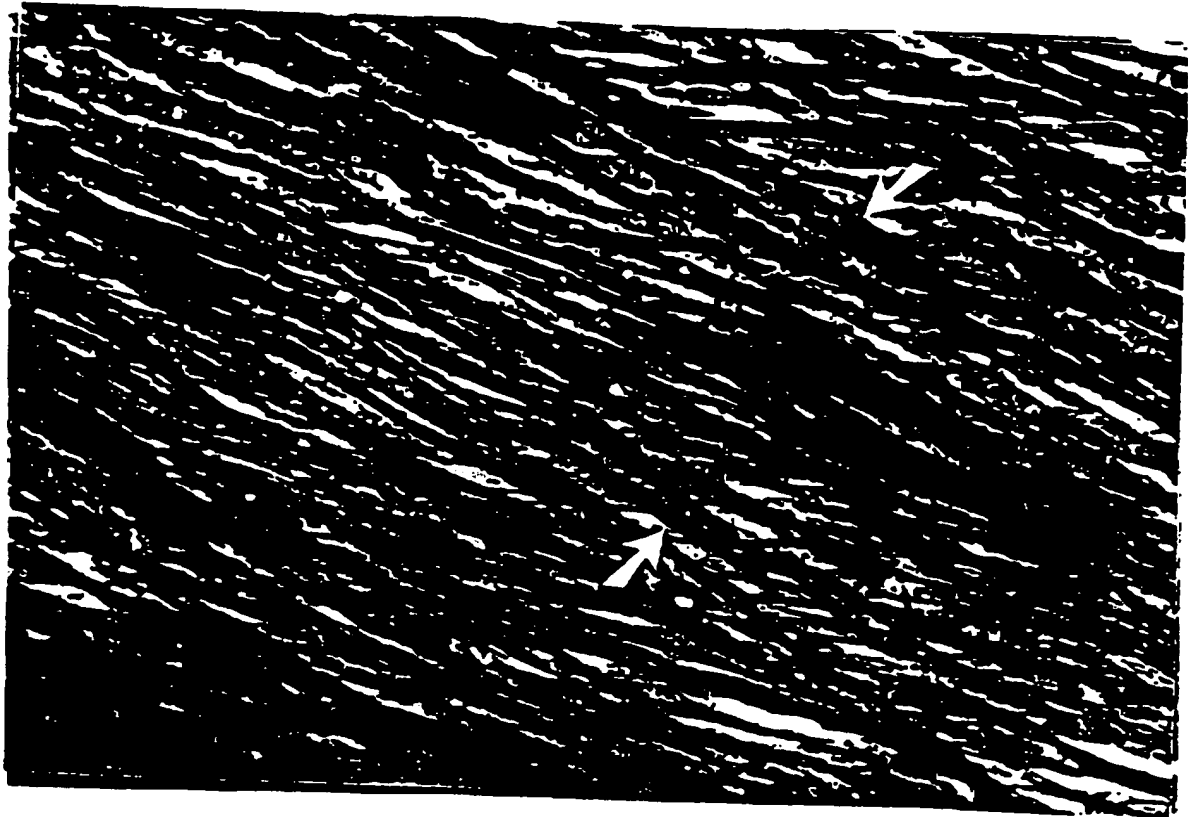
FIG 6. MYOBLAST VS MYOFIBER GENE THERAPY

PATIENT

Isolate Cells

MYOBLAST

Insert Gene for Bioactive Compound

Inject Myoblasts

Grow Myoblasts

Implant Tissue Specimen

MYOFIBER TISSUE SPECIMEN

TISSUE CULTURE

myofibt2.vsd
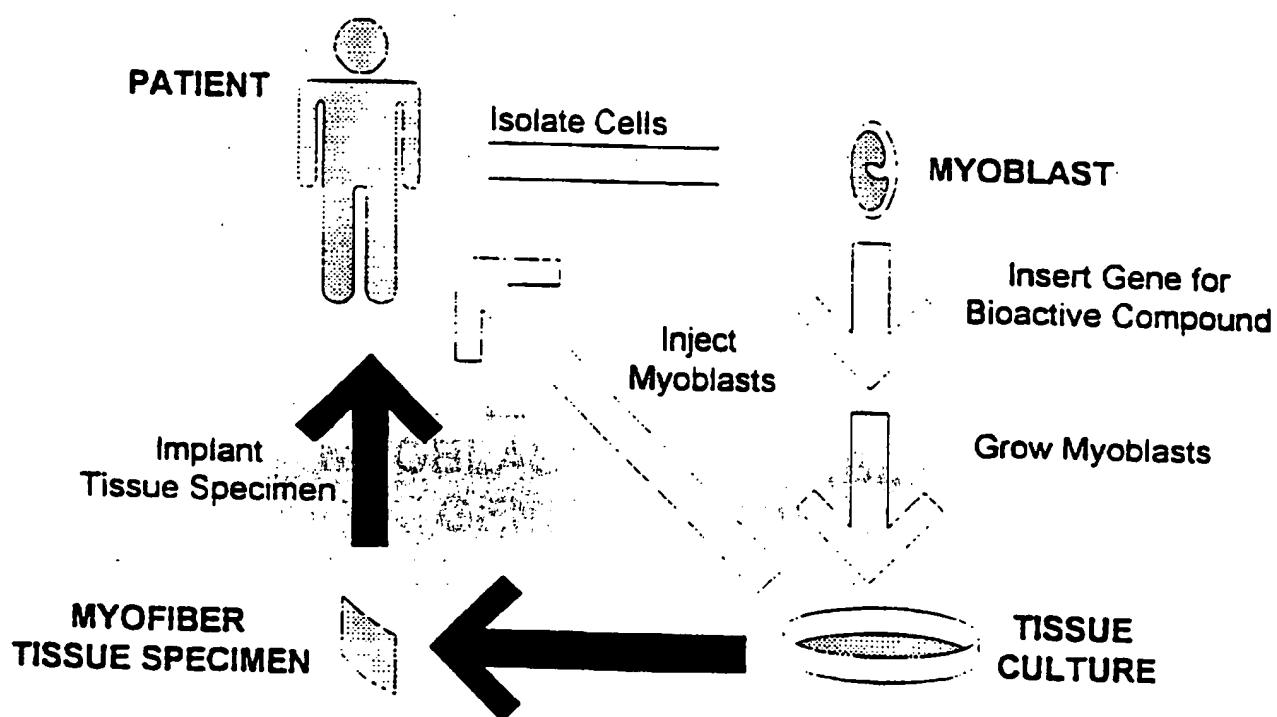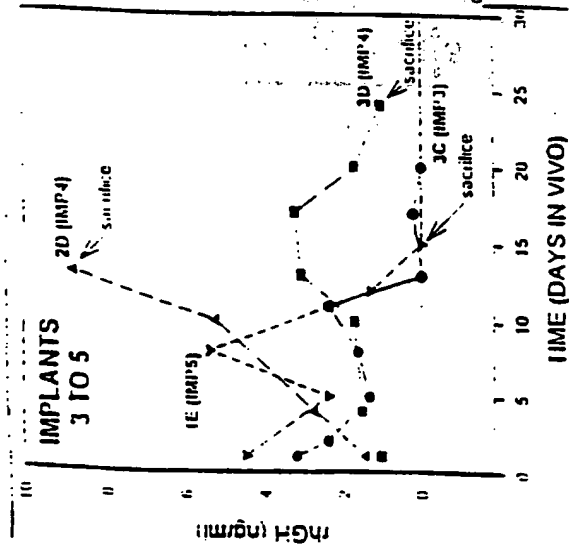
FIGURE 7A

Fig. 7B
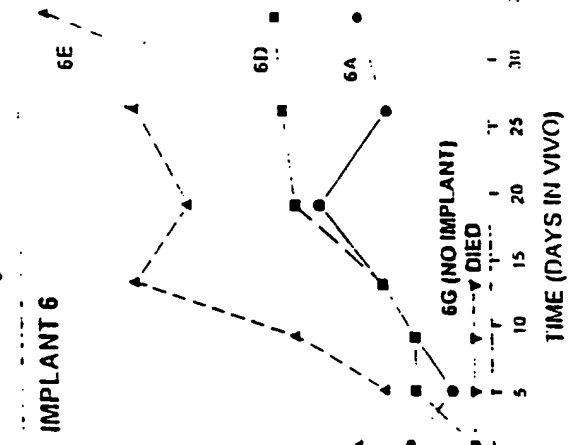
Fig 7C
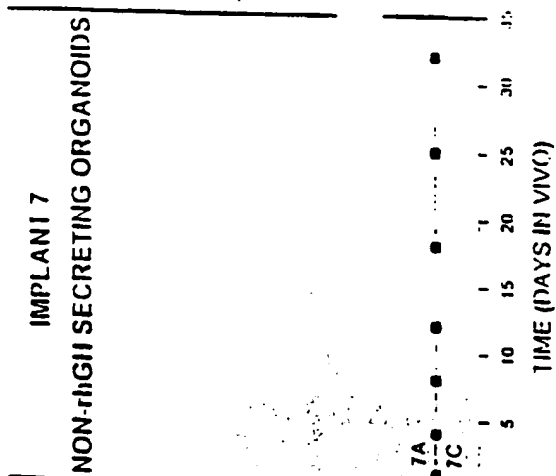
Fig. 7D

Fig. 7E
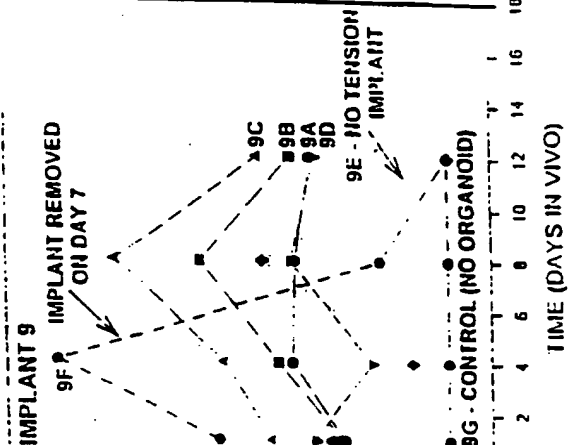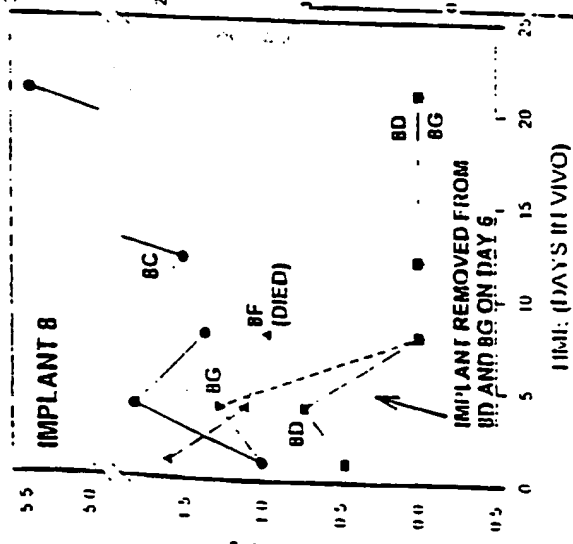
Fig 7F
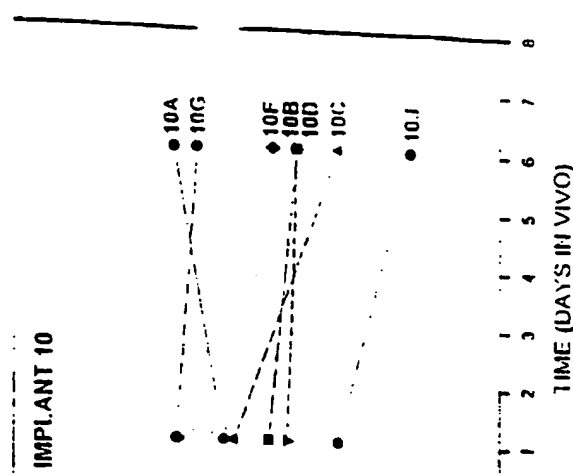
FIGURE 8. Cytosine arabinoside (1μg/ml) for 4 days kills greater than 99% of proliferating C2C12 myoblasts (left graph) but has little effect on postmitotic myofibers (right graph).
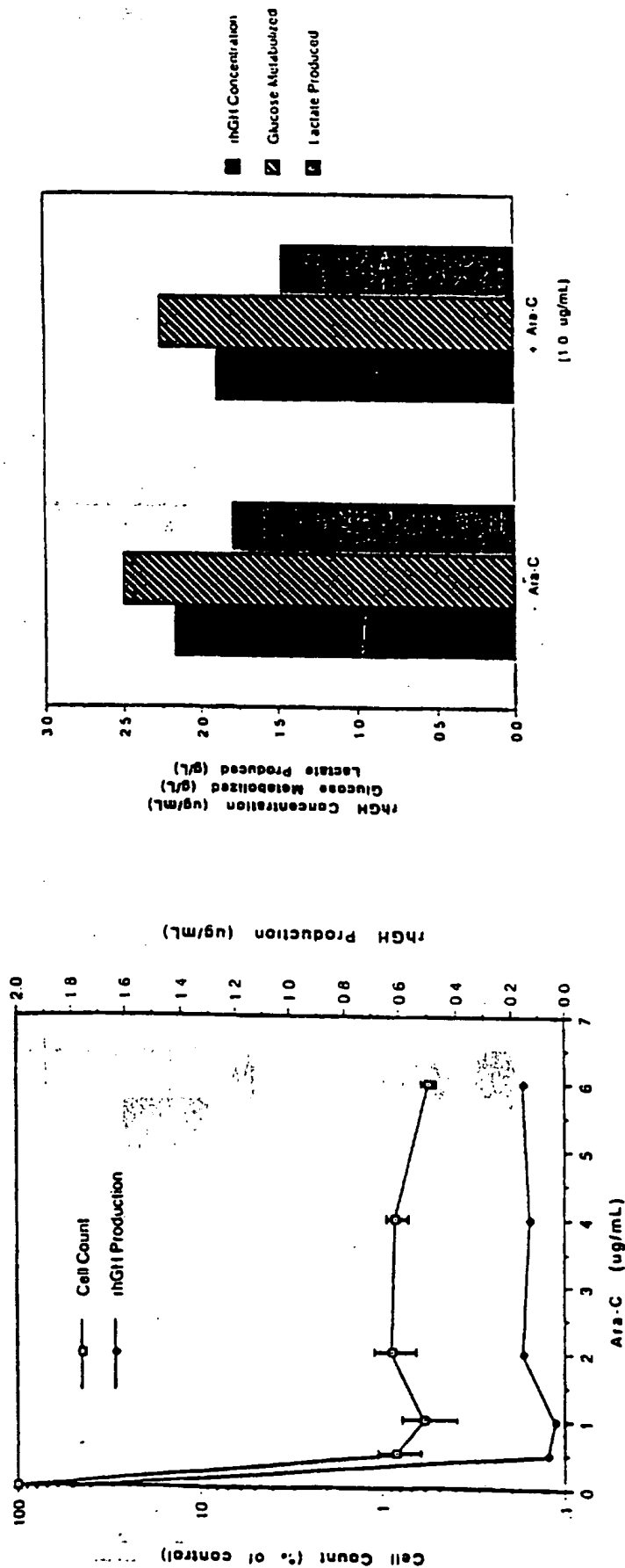
Fig. 8B

Fig. 8A

FIGURE 9. (A) rhGH ∴ creting muscle organ d removed after 2 weeks in mouse 2D; (B) H & E stained cryostat cross-section of organoid shown in (A), with well differentiated myofibers running longitudinally in the organoid, and parallel to each other (arrows); and (C) X-gal blue staining of β-galactosidase activity in the cells containing the rhGH gene and β-gal gene (co-transfected in the same C2C12 myoblasts).
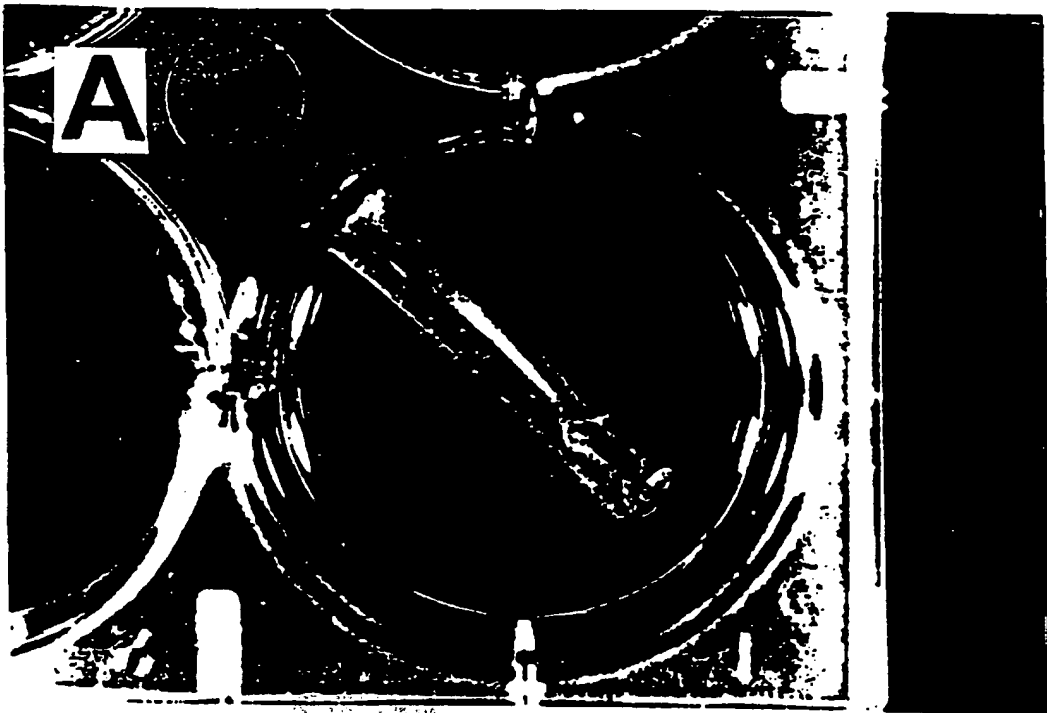
FIGURE 9. (con't)
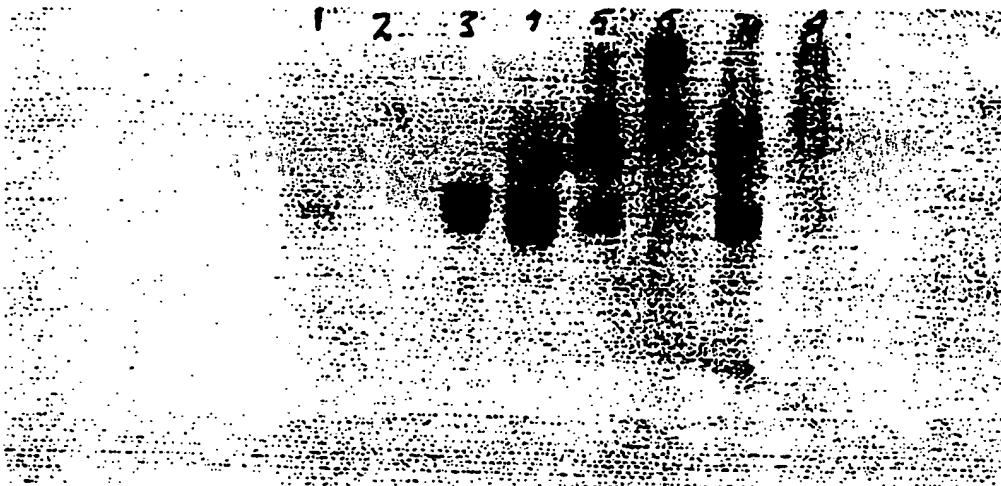
Figure 10A

Figure 10B

Figure 10C

Fig. 11
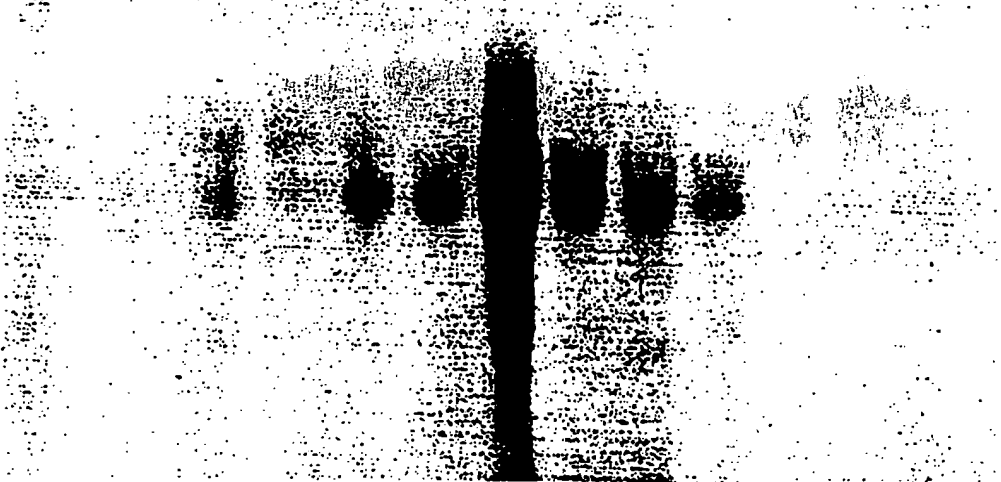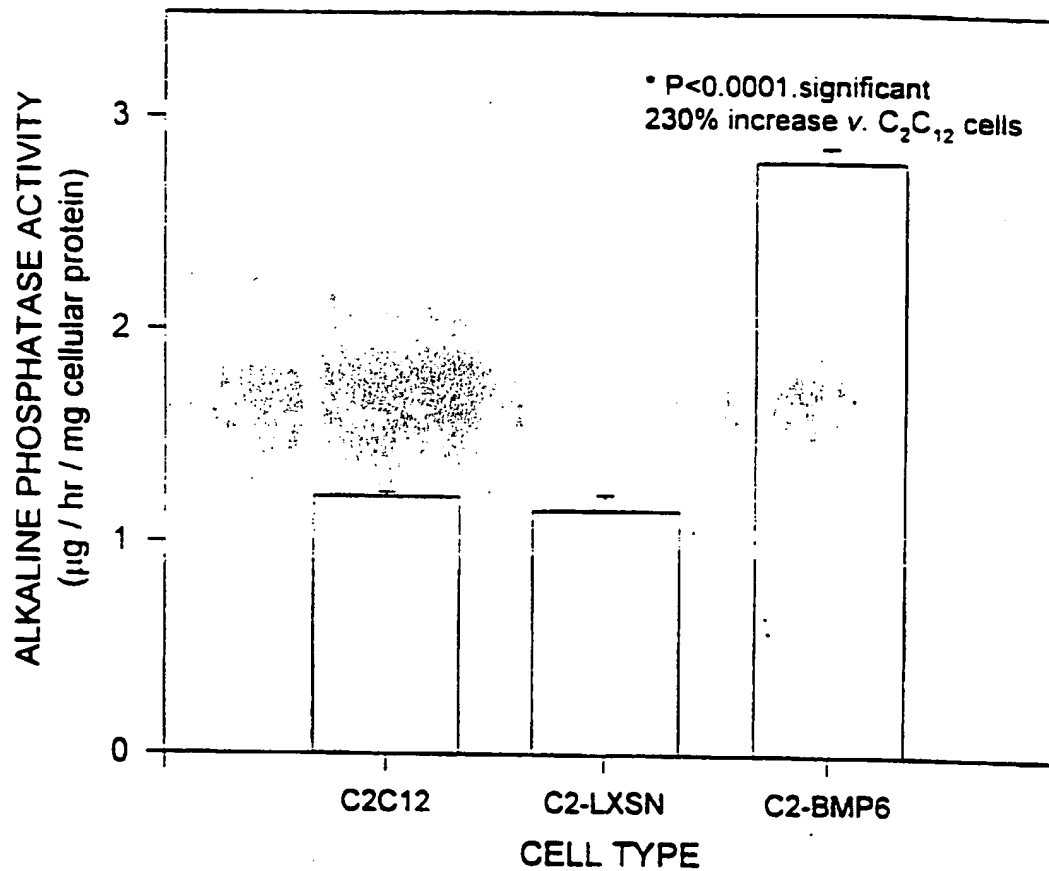
Cell Type	n	Alkaline Phosphatase Activity (μg/h/mg cellular protein) (mean ± st. err.)	Total Cellular Protein (mg) (mean ± st. err.)
C_2C_{12} cells	4	1.21 ± 0.02	0.391 ± 0.019
C_2-LXSN cells	4	1.15 ± 0.07	0.413 ± 0.018
C_2-BMP6 cells	4	2.79 ± 0.07	0.381 ± 0.016

Fig. 12 A

Fig. 12 B

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N5/16   A61K48/00   C12M3/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N   A61K   C12M

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 00439 A (UNIV BROWN RES FOUND) 7 January 1993 see the whole document ---	1-37
X	WO 93 21859 A (GIAMPAPA VINCENT CARMINE) 11 November 1993 see the whole document ---	1,19
X	US 4 940 853 A (VANDENBURGH HERMAN H) 10 July 1990 see the whole document ---	18-37
	-/--	

X	Further documents are listed in the continuation of box C.

X	Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 May 1997	2 7. 05. 97

| Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Authorized officer Hillenbrand, G

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 5, 1995, page 2099-2106 XP000652068 PERRONE, C.E. ET AL.: "Collagen and stretch modulate autocrine secretion of insulin-like growth factor-1 and insulin-like growth factor binding proteins from differentiated skeletal muscle cells" see abstract	1

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9300439 A	07-01-93	US	5182111 A	26-01-93
		AU	652071 B	11-08-94
		AU	2296192 A	25-01-93
		CA	2090719 A,C	29-12-92
		EP	0547203 A	23-06-93
		JP	6502080 T	10-03-94
WO 9321859 A	11-11-93	US	5326568 A	05-07-94
		AU	4370593 A	29-11-93
		CA	2134680 A	11-11-93
		EP	0637946 A	15-02-95
		JP	8500088 T	09-01-96
		US	5494677 A	27-02-96
		US	5580569 A	03-12-96
US 4940853 A	10-07-90	US	5153136 A	06-10-92